variation in the proportion of the products may be obtained by varying the conditions.

With regard to the results of exactly comparable experiments with mannitol, good evidence has been obtained in confirmation of the view already put forward by the writer that the fermentation of various carbohydrates and allied substances by bacteria is brought about by a single set of enzymes whose actions are common to all such cases of fermentation. This does not exclude the possibility that the first step in the degradation of a particular molecular structure may require a special enzyme in order to produce the first intermediate substance, which according to the writer's hypothesis would be the same for all analogous cases of fermentation.

The Enzymes Concerned in the Decomposition of Glucose and Mannitol by Bacillus coli communis. Part III.—*Various Phases in the Decomposition of Glucose by an Emulsion of the Organisms.*

By Egerton Charles Grey (Beit Memorial Research Fellow).

(Communicated by Dr. A. Harden, F.R.S. Received July 25, 1917.)

(From the Laboratory of Prof. A. Fernbach, Institut Pasteur, Paris.)

CONTENTS.

In Part II of this series results have been described which indicate the existence of several independent processes occurring during the one experiment. The experiment which will be now described was undertaken with the object of simplifying results by shortening the time of the fermentation. Arrangements were made also to count the bacteria at various periods, with the object of determining how far the fermentation was due to enzyme action which could be said to be carried on independent of the multiplication of the cells. The plan of the experiment was as follows:—About 50 grm. of glucose was to be fermented in a volume of 5 litres of solution. A sample of about a litre was to be removed every 12 hours and submitted

to analysis. By a comparison of these analyses the amounts of the various products which had been formed during each interval of time would be determined.

Certain modifications were necessary in the form of apparatus used and in regard to the analysis. With regard to the analysis, the estimation of carbon dioxide from time to time during the course of the experiment required a special device. In the first place, the gas space above the solution would gradually increase as the samples of solution were removed, and diffusion from the solution to the space above would cause the concentration of CO_2 in the solution to be constantly changing, and in the second place, since the sample of the fermentation solution would have to be removed while warm, precautions would have to be taken to avoid loss of CO_2 prior to its mixture with standard barium hydroxide solution. The apparatus was therefore arranged according to the plan represented in the figure below.

Arrangement of Apparatus for the Study of the Various Phases of a Fermentation.

The essential differences between the apparatus for the study of the fermentation in stages, and that (fig. 1 of Part II) for the case of a complete fermentation are—the flask A for the withdrawal of the samples of the solution ; the gas burette H for the removal of samples of gas and estimation of the carbon dioxide; and the arrangement of the three-way tap K by means of which the system of bottles, L′, L″, L‴, containing the alkali for the absorption of the carbon dioxide evolved during the fermentation, may be shut off from connection with the fermentation flask D, while the hydrogen which has collected in the reservoir M is returned to the fermentation flask to replace the volume of the liquid removed at A.

The method of operating is as follows:—The flask D at the beginning of the experiment contains about 5 litres of solution, and the air space above the liquid is about half a litre. The pinchcock C is closed, the taps H and K are closed, and F and G are opened. By the aspiration of the water-pump with which G is in connection, the flask D is deprived of air, the solution being brought to the boil under the reduced pressure. Nitrogen is introduced through the tube B after removal of the flask A. During the course of the fermentation the three-way tap K is turned so that the evolved gases pass through the bottles, L^1, L^2, L^3, into which a known amount of CO_2-free alkali has been introduced. The bottles are arranged so that they may be readily removed, and the amount of carbon dioxide absorbed determined.

In order to ascertain the amount of carbon dioxide which has been produced up to any moment of the fermentation, it is necessary to estimate the gas which has been absorbed by the alkali in the bottles, L′, L″, L‴, the amount which is present in the fermentation solution, and the amount which is in the gas space in the fermentation flask. These two last must be determined simultaneously owing to the possibility of repartition of the carbon dioxide between the two media by diffusion. The arrangement of the apparatus permits of this simultaneous determination. The taps F

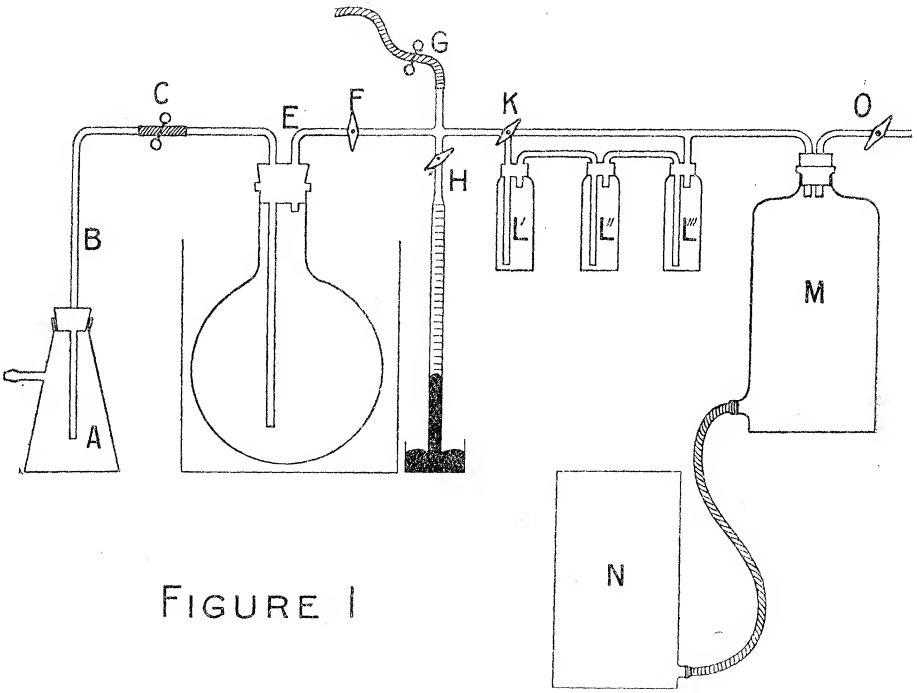


FIGURE 1

and K are closed, and H and G opened; mercury is thus drawn up into the burette; G is then closed. A sample of the solution in the flask is removed for the estimation of dissolved carbon dioxide by connecting an evacuated flask containing a quantity of standard barium hydroxide solution with the tube B and opening the pinchcock C, as described in the section on the estimation of carbon dioxide. At the instant that the sample of solution is removed, the tap F is opened, and a sample of gas thus drawn into the gas burette by the fall of the mercury. A small piece of solid potassium hydroxide is allowed to float up into the burette for the absorption of the carbon dioxide in the sample of gas.

Immediately after the sample of solution has been drawn off into the evacuated flask for the determination of the carbon dioxide dissolved, a much larger sample is withdrawn into the flask A, which is slipped over the tube B, which already supports a rubber stopper. To withdraw the sample the flask A is evacuated, and the tap K is turned so as to shut off the absorption bottles and put the fermentation flask into direct communication with the gas-holder M. The vessel N is raised. Now upon opening the cock at C the sample is drawn into A and the hydrogen and nitrogen in M are returned to the fermentation flask.

The hydrogen can be estimated either by the measurement of the change in volume of the gas or by direct determination. At the end of the experiment, when all the samples of solution have been removed from the fermentation flask, the gases may be displaced by the introduction of hot water through B, and collected in M.

The arrangement of apparatus is suitable for following the stages of either aërobic or anaërobic fermentations.

Details of an Experiment in which the Products of Fermentation have been Examined at Successive Stages.

The experiment was carried out as follows:—A weight of 45·84 grm. of pure anhydrous glucose was dissolved in distilled water and the solution sterilised. This solution was then added to about 3 litres of sterile distilled water in a flask of $5\frac{1}{2}$ litres capacity, in which also was about 40 grm. of chalk contained in small floating sacks. The sugar was thus not sterilised in contact with the chalk. The contents of the flask were cooled to 37° C., and 750 c.c. of an emulsion of bacteria was added. The emulsion of bacteria was made from the surface of agar by means of a solution of potassium and magnesium sulphates. The amount of each introduced finally into the solution to be fermented was 11·6 grm. of potassium sulphate and 3·3 grm. of magnesium sulphate. The total volume of the solution in the flask at the beginning of the experiment was 5100 c.c. The fermentation was allowed to take place at approximately 40° C. The weight of bacteria introduced corresponded to a dry weight of 0·61 grm., and the weight of other organic matter washed from the agar bottles was 1·83 grm.

The number of living bacteria introduced at the beginning of the experiment was $5100 \times 217 \times 10^{12}$ or 217×10^{12} for each cubic centimetre of the solution in the flask.

At the end of 12 hours from the time of introduction of the bacteria samples of the solution were removed for the estimation of the various

products resulting from the fermentation and also for the enumeration of the living bacteria. The operation was repeated at 24, 48 and 72 hours from the commencement of the experiment. The number of bacteria living was determined in each case by plating out on nutrient agar.

The experiment was thus divided up into four periods. The nature of the change during any period was ascertained by a comparison between the composition of the fermentation solution at the beginning and end of the period. The changes in the number of the bacteria and the extent to which the sugar was fermented during each period may be seen from the following figures.

Period.	Duration.	Number of bacilli per c.c. of the solution at the beginning of each period in milliards.	Amount of sugar at the start of the period.	Amount of sugar at the end of the period.	Amount of sugar changed during the period.	Volume of the solution during fermentation.
	hrs.		grm.	grm.	grm.	c.c.
1	12	217,000	45·84	35·06	10·78	5100
2	12	4·5	30·95	20·41	10·54	4400
3	24	1,400,000	16·79	10·09	6·70	3620
4	24	450,000	6·84	3·3	3·54	2214
	72					

The following facts may be noted. During the first period of 12 hours there is an enormous falling off in the number of living bacteria, and during the second period of 12 hours there is a still greater increase in the number which are living. Subsequently there is a diminution also but proportionally small as compared to the diminution during the first period. In spite of the fact that the first period is characterised by rapid diminution and the second period by rapid multiplication of the bacteria, nevertheless the amount of sugar transformed into products which do not reduce copper solution is approximately the same in both cases.

The sample which was removed for analysis at the end of the first 12 hours gave the following results. In the result must also be included the carbon dioxide evolved during this period.

Result of Analysis of the Fermentation Solution at the end of the First Period of 12 *Hours.*

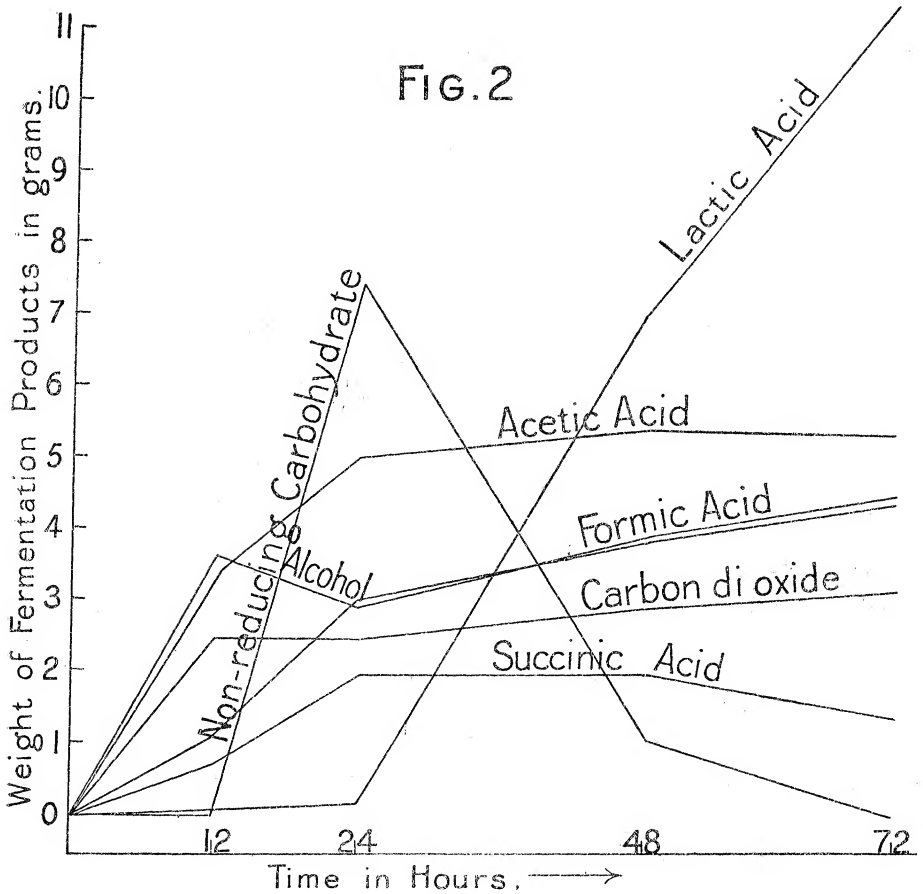
	grm.	grm.
Weight of sugar fermented		10·78
Products found—		
Carbon dioxide	2·43	
Formic acid	1·09	
Acetic acid	3·40	
Succinic acid	0·72	
Lactic acid	0·10	
Ethyl alcohol	3·56	
Hydrogen, 420 c.c. at N.T.P	0·04	
	——	11·34

Ratio CO_2/H_2 ..... 1·4

This result is of considerable importance, for it shows that during the period of death of the bacteria the production of lactic acid ceases. The fact that ethyl alcohol, acetic acid and (formic acid + carbon dioxide), each represent about a third of the sugar fermented, and that the proportion between these substances is the same as when the fermentation is carried out under very different conditions, speak strongly in favour of the view already put forward as to the origin of these substances in common from a single intermediate substance, and by the action of the same enzyme. The fermentation during the first period has in fact occurred in the manner which was to be expected from the experiments described in Part II provided it were possible to exclude the action of the lactic-acid-producing enzyme, a result which has here been achieved. It has been shown in Part II that succinic acid has an origin probably in common with acetic acid and that the two substances could replace one another; it requires therefore no change in the writer's hypothesis to account for the variations in the amount of succinic acid produced in these experiments.

Here, as in the earlier experiments of Harden and later also of the writer, the tendency is seen for the alcohol and acetic acid to be produced in equimolecular proportions.

In order to ascertain the proportion of the products formed during the subsequent periods of the fermentation, it is necessary to subtract from the amounts of the products found in each period the amounts of such substances which were present at the commencement of the period under consideration. These results may be deduced from a study of fig. 2, which is a graphical record of the rate of formation of the various products of the fermentation.



Products of the Fermentation of Glucose formed during the Second Period of 12 Hours.

	grm.	grm.
Weight of sugar fermented		10·54
Products found—		
Carbon dioxide	0·028	
Formic acid	1·895	
Acetic acid	1·620	
Succinic acid	1·158	
Lactic acid	0·16	
Ethyl alcohol	Nil.	
Non-reducing carbohydrate*	7·40	
		12·26
Hydrogen	about 50 c.c.	

* Calculated on the assumption that the reducing sugar formed on hydrolysis has the same reducing power as glucose.

Since the products add up to a greater weight than can correspond to the sugar fermented, it is possible that the weight of the carbohydrate synthetised by the bacteria cannot be quite accurately calculated from the reduction of copper solution after hydrolysis. It is also possible that the additional weight is derived from the organic matter introduced with the bacteria or formed during the autolysis of the bacteria in the first period.

But the conclusions which may be drawn from the analysis recorded above are not affected by the fact that the products obtained are somewhat in excess of the sugar used. The quantity of sugar which has been transformed or synthesised into a form in which it does not reduce copper solution until after hydrolysis represents about 70 per cent. of the original glucose.

The great importance of this result will be considered later. For the moment it will suffice to note the following facts with regard to this period of the fermentation.

It is essentially a period of synthesis. There is practically no production either of alcohol or carbon dioxide or hydrogen. There is, as in the first period, no formation of lactic acid. On the other hand, there is here a formation of succinic acid in greater proportion to the acetic acid than in the first period.

The products formed during the third period, *i.e.* during the second 24 hours of the fermentation, were determined, as in the case of the second period, by difference.

The results were as follows:—

Products of the Fermentation of Glucose formed during the Third Period (24 *to* 48 *Hours*).

	grm.	grm.
Weight of glucose fermented	6·70	
Weight of non reducing carbohydrate fermented	4·98	
	——	11·68
Products found—		
Carbon dioxide	0·34	
Formic acid	0·82	
Acetic acid	0·46	
Succinic acid	0·02	
Lactic acid	6·63	
Ethyl alcohol	1·05	
	——	9·32

Hydrogen not estimated.

It will be seen that there is a loss here which almost corresponds in

magnitude to the gain of the previous experiment and that the two results taken together would balance. This seems to indicate that the error lies in the determination of the residual glucose by the reduction of copper.

The most striking fact observed in the result of the third period of fermentation is that it is mainly a lactic acid production; 60 per cent. of the carbohydrate fermented has been transformed into lactic acid.

The synthetic period during which there is practically no lactic acid formed is immediately followed by a degradation process both of the sugar in the solution and also the carbohydrate previously elaborated. This degradation period is characterised by the formation of a very high yield of lactic acid, which in part, at least, comes from the carbohydrate previously synthesised. The production of alcohol, which had ceased during the period of rapid growth and multiplication of the bacteria, viz., the period of synthesis, commences again in the ensuing period of degradation. The second period of alcohol production, like the first, is a period of death for the bacteria.

Products of the Fermentation of Glucose formed during the Third 24 *Hours of the Fermentation.*

	grm.	grm.
Weight of glucose fermented	3·41	
Weight of non-reducing carbohydrate fermented	0·67	
	——	4·08
Products found—		
Carbon dioxide	0·37	
Formic acid	0·62	
Acetic acid	Nil.	
Succinic acid	Nil.	
Lactic acid	2·86	
Ethyl alcohol	0·39	
	——	4·24

Hydrogen not estimated.

It will be seen that the fermentation that has taken place during this interval is in the main the same as during the preceding interval of 24 hours. The whole period is chiefly characterised by the production of lactic acid.

The excess of formic acid over alcohol is not in keeping with the results of previous analyses, for it has always been found that the ratio of alcohol and acetic acid to formic acid is a constant,* but there are reasons to doubt the correctness of this formic acid figure obtained here, since it was found that the solution of sodium formate gave a precipitate of iodoform in the cold on

* Grey, 1914.

the addition of sodium hydroxide and iodine, and it is possible that the substance causing this reaction may also have reduced mercuric chloride. It was observed that the precipitate of mercurous chloride obtained in this experiment in the estimation of formic acid was blackened. The figures for this fourth analysis are derived by the consecutive subtractions from the previous analyses, and it would therefore not be safe to insist too much upon small differences here, especially as the total amount of sugar fermented during this last interval is small.

It is against the principles indicated in the commencement of this communication to prolong the period of a fermentation beyond the time during which the main primary changes occur, and in the discussion of these results therefore conclusions will alone be considered in as far as such can be supported by the evidence of the first 48 hours. Even this period is far too long and it will be the object of future researches to reduce it.

The balance sheet of this experiment is made out in the following Table.

Table II.—Balance Sheet for the Experiment on the Phases of the Decomposition of Glucose by *B. coli communis.*

Time from the beginning.	Carbohydrate decomposed.		Products recovered.	
	Glucose.	Non-reducing carbohydrate.	Of degradation.	Of synthesis.
hours.				
12	10·78	—	11·30	—
24	10·54	—	4·86	7·40
48	6·70	4·98	9·32	—
72	3·41	0·67	4·08	—
	31·43	5·65	29·56	7·40
	37·08		36·96	

For the sake of easier comparison the results are grouped together in Table III, the products being represented as percentage of the total weight of products of degradation in each period. The balance sheet shows that there is no total loss during these four parts of the experiment, but, owing to the difficulty of being certain as to the amount of glucose as determined by reduction of copper solution, it may be that the proportion between reducing and non-reducing carbohydrate during the intermediate periods is not of the same order of accuracy as the determination of the ultimate degradation products. Since, however, the non-reducing substance produced

during the second 12 hours is subsequently degraded, it does not in the end change the results.

Table III.—The Results of the various Periods of the Fermentation expressed as Percentages of the Amount of Total Products obtained in each Period, ignoring the Temporary Formation of Non-reducing Carbohydrate.

	1.	2.	3.	4.
Carbon dioxide	21·50	0·50	3·70	8·70
Formic acid	9·61	39·00	8·80	14·60
Alcohol	31·50	Nil.	11·30	9·20
Acetic acid	30·10	33·40	4·90	Nil.
Succinic acid	6·37	23·80	0·20	Nil.
Lactic acid	0·88	3·30	71·10	67·50

The results have been calculated to the amount of total products obtained in the period rather than upon the weight of sugar fermented during the period owing to the presence of the non-reducing carbohydrate, *i.e.*, carbohydrate which does not reduce copper solution until after hydrolysis. As has been pointed out, it is not possible to be sure of the reduction figure. Moreover, the object of the above Table is to bring out the comparison between the degradation processes occurring during each period, and this comparison is only obscured by the calculation of the results to the sugar which has disappeared, since part of the sugar becomes synthesised into the non-reducing carbohydrate which later undergoes degradation.

General Considerations.

Before discussing the broad conclusions which may be drawn from these researches, the writer would put forward certain considerations with regard to the methods which these results, and the results of previous work also, suggest should be adopted in future work on bacterial fermentation. These suggestions are put forward partly because this branch of chemistry is essentially one in which the co-operation of several workers is needed, and also because the results obtained by adopting the method of research described in this communication warrant the conclusion that a more extended application of these methods would help to elucidate the problems of bacterial enzyme action. With comparatively few experiments it has been possible to show that the enzymes of *B. coli communis* are, partly at least, independent of one another in their action; that the degradation of glucose is brought about by means of these independent enzyme fermentations acting either simultaneously or consecutively, and that the same set of enzymes produced by the bacterium serve for the degradation of substances allied to glucose, such as mannitol. It has been shown also

that the nature of the fermentation products, and the proportion in which these appear in the final analysis, will depend on the extent to which the various enzyme actions co-operate, which in turn depends on conditions such as concentration of salts and temperature. It has been shown also that the synthetic side of the process of bacterial fermentation is quantitatively of the same order as the decompositions which follow.

Some of these facts have so far been hidden because the various changes occur rapidly, and previous experiments have been too prolonged to serve for their investigation. In the light of these results it is of interest to consider what the objections are to prolonged fermentations if such experiments aim at a characterisation of the enzymes of any particular organism at any particular time. For it is important to insist that a organism is not constant as regards the enzymes it contains, but that its composition in this respect will depend upon its immediate past history, and thus only a superficial idea of the fermentation processes set up by the organism is given by a study of the action as a whole, and it is necessary, in order that any true uniformity should be obtained, that the actions of the separate enzymes should be studied.

The objections are as follows:—

(1) The strain of the organism introduced at the start may vary with the production of new enzymes.

(2) Even if the enzymes remain constant in kind and amount their actions may be selectively impeded by the conditions of the experiment at the start, or by the accumulation of the products of the fermentation.

(3) Even if the enzyme actions were unimpeded by the conditions imposed, the prolongation of the experiment would give nothing but the sum of the various actions concerned.

For, if an organism be introduced in a small seeding into an artificial medium, the enzymes which are developed in the subsequent generations of the organism may not necessarily be the same as those which were present at the start. There may be adaptation to the conditions in the sense of the production of enzymes which bring about the decomposition of the substance under investigation along simpler lines. As an example of such a selection taking place during a single fermentation experiment, it will suffice to refer to an observation made by the writer in an earlier communication, namely, that after the fermentation of mannitol by certain strains of *B. coli communis* had come to a standstill as regards the production of gas, the strain which could now be isolated from the solution differed from the original strain in that it now no longer produced gas from mannitol even when inoculated into a fresh solution.

Again, although the strain introduced at the beginning of the experiment should remain constant during subsequent generations, as regards the production of enzymes, yet the action of these enzymes would be modified by the progressive change in the conditions resulting from the accumulation of the products of their action. If such products affected equally all the enzymes, then the result might simply be a slowing down of the fermentation process as a whole, but if, as is more likely, the enzymes are differently influenced, it may be that the course of the fermentation would vary from time to time if a prolonged fermentation experiment were made. Grimbert has shown this to be the case with a bacillus producing butyric acid, and Fernbach and Schoen have shown that the course of yeast fermentation can be diverted into channels wherein there is a large production of acids, especially interesting amongst which is pyruvic acid.

It may perhaps be pertinent to remark in this connection that in all experiments in which chalk or other alkali is added to the medium there is probably a tendency to the encouragement of the action, if not of the production, of acid-forming enzymes.

Moreover, even if the enzymes introduced at the start remained constant throughout the experiment and their actions were not selectively impeded by the products of the fermentation or by other conditions, the experiment would not serve to distinguish the products of one enzyme action from those of another. To do this it is necessary to know whether the various enzyme actions occur simultaneously or consecutively. In the first case, in order to study one of the actions, it may be helpful to be able to arrange the conditions so that the other reactions are brought to a standstill, and, in the second case, it may be that by reducing the period during which the fermentation is studied, it is possible to separate the various phases from one another. Both of these methods have been successful in the experiment described in this communication. During the first period the conditions of the experiment were accidentally such that the lactic acid enzyme did not act, and it was possible therefore to observe which substances were produced independent of it, and during the second 12 hours of the experiment it was possible to observe the importance of the synthetic side of the fermentation process, this period being really the period of rapid growth of the organism.

The method of research here described indicates the means by which the subject of the enzymes of bacteria can be more closely studied than has been hitherto possible.

Summary and General Conclusions.

By analysis of the products resulting from the decomposition of glucose by *B. coli communis*, it has been shown that the fermentation is made up of

several phases which can be separately studied if the period during which the experiment is made is sufficiently short. These phases are largely to be correlated with increase and diminution in the number of living cells which are present at any time.

It has been shown that during the period which was characterised by rapid death of the cells, there was no formation of lactic acid, whereas during a period which immediately followed the rapid multiplication of the cells, lactic acid was produced to the extent of 70 per cent. of the sugar consumed. The period in which the cells died was characterised by the transformation of sugar into alcohol, and formic, acetic, and succinic acids.

Also it has been shown that at this period sugar is decomposed to a greater extent than during the period of extensive multiplication, which points to the conclusion that the fermentation is brought about by enzymes.

During the period of multiplication there is a transformation of glucose into a more complex substance which only reduces copper solution after hydrolysis. The extent of the synthesis during this period is quantitatively of the same order as the degradation which is brought about in the period which follows.

From these results the following general conclusions may be drawn:—

Elaboration of glucose into more complex material may occur during certain phases of the life of bacteria to an extent exceeding the degradation brought about during the same time, so that it were a fallacy to suppose that bacteria are as a class concerned chiefly with the business of decomposition. The degradations which these organisms set up are the sequel of those processes of synthesis which are more immediately associated with the growth and multiplication of the cells. In 48 hours a synthesis of 3 grm. of fat has been observed when less than a gramme of bacteria was mixed with glucose under conditions unfavourable for the normal fermentation process, and during a period of 12 hours accompanied by rapid cell multiplication, a transformation of 7 grm. of glucose into a more complex carbohydrate has been obtained. Thus it will be seen that the synthetic side of the fermentation process brought about by bacteria has been hidden because of the rapidity with which the processes of degradation supervene. In order to study the process of synthesis the fermentation must be interrupted as a rule before 24 hours have elapsed.

The second general observation which may be made is with regard to the existence of enzymes in bacteria. No one has doubted, since Buchner's demonstration of the existence of zymase in yeast and of certain other enzymes in bacteria, but that the fermentation processes brought about by bacteria and other organisms were the result of enzyme action.

Nevertheless, the independent existence within the cell of enzymes (if by definition such enzymes act independently of the life of the cell) has been, in the case of bacteria at least, rather a matter of speculation.

It may be suggested here, however, that it is not necessary to isolate from cells an unorganised material capable of bringing about a fermentation in order to demonstrate that such a fermentation is brought about by enzymes (unless such substances were defined as enzymes only provided they could be isolated by the present means at our disposal). If an enzyme is regarded as a substance capable of inducing fermentation independently of the life of the cell, then there are two methods of demonstration which serve to establish the existence of enzymes in any particular case without the necessity of separating them from the cell. The first method consists in carrying out the fermentation under conditions which do not support the life of the organism. The first part of the experiment described in this communication very closely approaches such conditions. Here it was shown that the amount of sugar decomposed during the rapid diminution in the number of living cells was as great as during that period where the number of living cells was both at the start and the finish enormously greater.

The second method, it is suggested, of demonstrating without actual destruction of the cell that the fermentation is brought about by enzymes, depends upon the proof that the several fermentation phenomena are independent of one another. For if a series of functions of a cell are absolutely independent of one another, some of them, at least, cannot be essential to the life of the cell.

Both methods here referred to have found application in the experiments described in this communication, and, although the separation of the phases of the fermentation was not absolute, either as regards complete absence of living cells at any one time, or complete transformation of glucose in one direction only, nevertheless taken in conjunction with the earlier work of Harden and Penfold, and later of the writer, the present results leave little room for doubt that the several fermentation processes by means of which *B. coli communis* brings about the decomposition of glucose and allied substances are true enzyme actions, and are capable of acting independently of one another, and thus breaking down the sugar in various ways.

This conclusion is probably applicable to other cases of fermentation.

In conclusion I would express my thanks to Prof. Auguste Fernbach for his kind hospitality and for valuable criticism.